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RPP Tracking #: RPP-04-214

Appendix A:

**USAMRIID FORM 360-1-1
CLEARANCE OF PUBLICATIONS & PRESENTATIONS**

Date: January 6, 2004

Submitting Author: David DeShazer, Ph.D.

Title: Genomic Diversity of *Burkholderia pseudomallei* Clinical Isolates: Subtractive Hybridization Reveals a Temperate Bacteriophage Present in *B. pseudomallei* 1026b

Submission Information:

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Research Plan/Project Number: 02-4-5X-026

Animal Use Protocol Number: B03-02 and B99-06

Human Use Protocol Number:

CDC Select Agent(s) Described: *Burkholderia pseudomallei* and *Burkholderia mallei*

Submitting Author Acknowledgement (for USAMRIID-sponsored work):

I acknowledge that the information contained in this submission accurately reflects the content of the proposed publication or presentation, and that I have given proper consideration to authorship and intellectual property issues.

David DeShazer

Report Documentation Page		Form Approved OMB No. 0704-0188
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		5e. TASK NUMBER
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14. ABSTRACT Burkholderia pseudomallei is the etiologic agent of the disease melioidosis and is a category B biological threat agent. The genomic sequence of B. pseudomallei K96243 was recently determined, but little is known about the overall genetic diversity of this species. Suppression subtractive hybridization was employed to assess the genetic variability between two distinct clinical isolates of B. pseudomallei, 1026b and K96243. Numerous mobile genetic elements, including a temperate bacteriophage designated phi1026b, were identified among the 1026b-specific suppression subtractive hybridization products. Bacteriophage phi1026b was spontaneously produced by 1026b, and it had a restricted host range, infecting only Burkholderia mallei. It possessed a noncontractile tail, an isometric head, and a linear 54,865-bp genome. The mosaic nature of the phi1026b genome was revealed by comparison with bacteriophage phiE125, a B. mallei-specific bacteriophage produced by Burkholderia thailandensis. The phi1026b genes for DNA packaging, tail morphogenesis, host lysis, integration, and DNA replication were nearly identical to the corresponding genes in phiE125. On the other hand, phi1026b genes involved in head morphogenesis were similar to head morphogenesis genes encoded by Pseudomonas putida and Pseudomonas aeruginosa bacteriophages. Consistent with this observation, immunogold electron microscopy demonstrated that polyclonal antiserum against phiE125 reacted with the tail of phi1026b but not with the head. The results presented here suggest that B. pseudomallei strains are genetically heterogeneous and that bacteriophages are major contributors to the genomic diversity of this species. The bacteriophage characterized in this study may be a useful diagnostic tool for differentiating B. pseudomallei and B. mallei, two closely related biological threat agents.		

15. SUBJECT TERMS Burkholderia mallei, pseudomallei, glanders, melioidosis, gemetic diversity, subtractive hybridization, bacteriophage					
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a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			



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[Name]

Co-Author Acknowledgement:

I am aware that I am listed as a co-author on the above-named submission, and I have reviewed the information contained in the enclosed publication or presentation and agree with its content.

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Editor Review:

The enclosed submission has been reviewed for technical, grammatical, and stylistic format. As appropriate, content has been verified to be identical to that contained in the previous submission referenced by the RPP Tracking Number.

Katherine Kendra 06 Jan 04
[Name & Date]

Animal Use:

The enclosed submission has been reviewed for compliance with the approved animal protocol cited above by a Veterinary Medicine Division Lab Animal Veterinarian.

S. D. Goodwin LTC, VC 7 Jan 04
[Name & Date] Susan D. Goodwin

Human Use:

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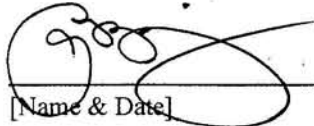
() Research does not involve Human Subjects.

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The enclosed publication or presentation has been reviewed for scientific quality and is consistent with the objectives of the Research Plan referenced above.

 14 JAN 04

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The enclosed submission for clearance is approved for release from USAMRIID.

 20 Jan 04

[Name & Date]

Hoff, Kay L Ms USAMRIID

From: Cavazos, Jaime Mr MEDCOM HQ
Sent: Thursday, January 22, 2004 9:12 AM
To: Hoff, Kay L Ms USAMRIID
Subject: RE: MANUSCRIPT RPP-04-214

Approved for publication.

Jaime Cavazos, SGM Ret.
Media Relations Officer
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-----Original Message-----

From: Hoff, Kay L Ms USAMRIID
Sent: Wednesday, January 21, 2004 8:56 AM
To: Cavazos, Jaime Mr MEDCOM HQ
Cc: Dasey, Chuck F Mr USAMRMC
Subject: FW: MANUSCRIPT RPP-04-214

Jaime,

Manuscript entitled "Genomic Diversity of Burkholderia pseudomallei Clinical Isolates: Subtractive Hybridization Reveals a Temperate Bacteriophage Present in B. pseudomallei 1026B" submitted for approval. The manuscript will be submitted to the Journal of Bacteriology, if approved. Thanks. Kay Hoff

-----Original Message-----

From: DeShazer, David Dr USAMRIID
Sent: Wednesday, January 21, 2004 9:41 AM
To: Hoff, Kay L Ms USAMRIID
Subject: RE: MANUSCRIPT RPP-04-214

<< File: manuscript1.doc >>

From: Hoff, Kay L Ms USAMRIID
Sent: Wednesday, January 21, 2004 9:37 AM
To: DeShazer, David Dr USAMRIID
Subject: MANUSCRIPT RPP-04-214

Dr. DeShazer;

Please send me an electronic copy of your manuscript entitled "genomic Diversity of Burkholderia pseudomallei Clinical Isolates: Subtractive Hybridization Reveals a Temperate Bacteriophage Present in B. pseudomallei 1026B". Your tracking number is RPP-04-214. Thank You. Kay Hoff

Kay Hoff, Program Assistant

Research Plans and Programs

(301-619-2767)

Genomic Diversity of *Burkholderia pseudomallei* Clinical Isolates: Subtractive Hybridization Reveals a *Burkholderia mallei*-Specific Prophage in *B. pseudomallei* 1026b

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Received 9 January 2004/Accepted 8 March 2004

Burkholderia pseudomallei is the etiologic agent of the disease melioidosis and is a category B biological threat agent. The genomic sequence of *B. pseudomallei* K96243 was recently determined, but little is known about the overall genetic diversity of this species. Suppression subtractive hybridization was employed to assess the genetic variability between two distinct clinical isolates of *B. pseudomallei*, 1026b and K96243. Numerous mobile genetic elements, including a temperate bacteriophage designated ϕ 1026b, were identified among the 1026b-specific suppression subtractive hybridization products. Bacteriophage ϕ 1026b was spontaneously produced by 1026b, and it had a restricted host range, infecting only *Burkholderia mallei*. It possessed a noncontractile tail, an isometric head, and a linear 54,865-bp genome. The mosaic nature of the ϕ 1026b genome was revealed by comparison with bacteriophage ϕ E125, a *B. mallei*-specific bacteriophage produced by *Burkholderia thailandensis*. The ϕ 1026b genes for DNA packaging, tail morphogenesis, host lysis, integration, and DNA replication were nearly identical to the corresponding genes in ϕ E125. On the other hand, ϕ 1026b genes involved in head morphogenesis were similar to head morphogenesis genes encoded by *Pseudomonas putida* and *Pseudomonas aeruginosa* bacteriophages. Consistent with this observation, immunogold electron microscopy demonstrated that polyclonal antiserum against ϕ E125 reacted with the tail of ϕ 1026b but not with the head. The results presented here suggest that *B. pseudomallei* strains are genetically heterogeneous and that bacteriophages are major contributors to the genomic diversity of this species. The bacteriophage characterized in this study may be a useful diagnostic tool for differentiating *B. pseudomallei* and *B. mallei*, two closely related biological threat agents.

Burkholderia pseudomallei is the causative agent of the glanders-like disease melioidosis (21, 22, 67). This organism is endemic in Southeast Asia and northern Australia, where it can be isolated from moist soil and surface water. Humans and animals can be infected by *B. pseudomallei* by direct inoculation of soil or water into skin abrasions or by inhalation of contaminated material. Underlying diseases such as diabetes mellitus and chronic renal failure are risk factors for melioidosis, but apparently healthy individuals can also develop clinical melioidosis (18). The clinical manifestations of melioidosis are protean and often include fever and abscess formation. The clinical spectra of melioidosis in endemic regions are similar, but brainstem encephalitis and genitourinary infections are more common in northern Australia while suppurative parotitis is more common in Southeast Asia (21, 22, 67). The basis for geographic differences in disease presentation is currently unknown, but the differences may be due to genetic differences in patients and/or in the *B. pseudomallei* strains present in the different regions.

Capsular polysaccharide and lipopolysaccharide (LPS) O antigen are important for *B. pseudomallei* virulence in animal models of melioidosis (4, 24, 55). The recently completed genome sequence of *B. pseudomallei* K96243 (<http://www.sanger.ac.uk/>) has facilitated identification of several new virulence gene candidates. In particular, K96243 harbors multiple

genomic islands with relatively low G+C contents, suggesting that there was recent acquisition by lateral gene transfer (34, 35, 49, 50). Lateral gene transfer is a process in which genetic material is transferred from a donor to a recipient via mobile genetic elements, such as plasmids, transposons, integrons, or bacteriophages. The laterally acquired genetic material can alter the phenotype of the recipient and promote adaptation to its environment. Further studies are required to elucidate the biology of *B. pseudomallei* mobile genetic elements and to examine their contribution to genomic diversity, niche adaptation, and virulence.

The goal of this study was to examine the genomic diversity of *B. pseudomallei* clinical isolates by performing subtractive hybridization between *B. pseudomallei* 1026b (tester) and K96243 (driver). *B. pseudomallei* 1026b was isolated in Thailand from a human case of septicemic melioidosis with skin, soft tissue, and spleen involvement and has been studied extensively in the laboratory (26). In this study, numerous mobile genetic elements in 1026b that were not present in K96243 were identified. One of the 1026b-specific mobile genetic elements was a temperate bacteriophage (ϕ 1026b) that was spontaneously produced during growth in liquid broth. The morphology, host range, genomic sequence, and immunological reactivity of bacteriophage ϕ 1026b are reported here.

MATERIALS AND METHODS

Bacterial plasmids, strains, and growth conditions. The plasmids used in this study are described in Tables 1 and 2. The *Burkholderia mallei* strains used in this study are listed in Table 3. The following *B. pseudomallei* strains were used in this

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics ^a	Source or reference
pCR2.1-TOPO	3.9-kb TA cloning vector; pMB1 ori; Km ^r Ap ^r	Invitrogen
pSKM11	Positive selection cloning and suicide vector; IncP <i>oriT</i> ; ColE1 <i>ori</i> ; Ap ^r Tc ^s	47
pGEM-7zf(+)	Standard cloning vector; pMB1 <i>ori</i> ; Ap ^r	Promega
pMOLUC	Vector for cloning large DNA fragments; pBR322 <i>ori</i> ; Ap ^r	28
pDD80	pGEM-7zf(+) containing 3,625-bp HindIII fragment from ϕ 1026b	This study
pDD81	pGEM-7zf(+) containing 3,105-bp HindIII fragment from ϕ 1026b	This study
pDD82	pGEM-7zf(+) containing 1,068-bp HindIII fragment from ϕ 1026b	This study
pDD83	pGEM-7zf(+) containing 602-bp HindIII fragment from ϕ 1026b	This study
pDD84	pGEM-7zf(+) containing 3,791-bp HindIII fragment from ϕ 1026b	This study
pDD85	pGEM-7zf(+) containing 2,515-bp HindIII fragment from ϕ 1026b	This study
pDD86	pGEM-7zf(+) containing 8,411-bp HindIII fragment from ϕ 1026b	This study
pDD87	pGEM-7zf(+) containing 9,355-bp HindIII fragment from ϕ 1026b	This study
pDD88	pMOLUC containing 12,775-bp HindIII fragment from ϕ 1026b	This study
pDD94	pSKM11 containing 930-bp internal fragment of ϕ 1026b gene 59 generated by PCR with primers MFS-2 and MFS-3; Ap ^r Tc ^r	This study
pDD101	pCR2.1-TOPO containing 126-bp HindIII fragment from ϕ 1026b	This study
pDD5025B	pSKM11 containing 9,492-bp HindIII fragment from ϕ 1026b; Ap ^r Tc ^r	This study

^a Km^r, kanamycin resistant; Ap^r, ampicillin resistant; Tc^s, tetracycline sensitive; Tc^r, tetracycline resistant.

study: 316c, NCTC 4845, 1026b, WRAIR 1188, USAMRU Malaysia 32, Pasteur 52237, STW 199-2, STW 176, STW 115-2, STW 152, STW 102-3, STW 35-1, K96243, 576a, 295, 296, 503, 506, 112c, 238, 423, 465a, 776, 439a, 487, 644, 713, 730, E8, E12, E13, E24, E25, E40, E203, E210, E214, E215, E250, E272, E277, E279, E280, E283, E284, E300, E301, E302, and E304 (3, 21, 23, 29, 31, 61, 70). *Burkholderia thailandensis* strains E27, E30, E32, E96, E100, E105, E111, E120, E125, E132, E135, E202, E251, E253, E254, E255, E256, E257, E258, E260, E261, E263, E264, E266, E267, E275, E285, E286, E290, E293, E295, and E299 (7, 61, 71) were also utilized in this study. Other *Burkholderia* strains used in this study included *Burkholderia cepacia* LMG 1222 (44), *Burkholderia multivorans* C5568, *B. multivorans* LMG 18823 (44), *Burkholderia cenocepacia* LMG 18863 (44), *B. cenocepacia* 715j (46), *Burkholderia stabilis* LMG 07000, *Burkholderia vietnamiensis* LMG 16232 (44), *B. vietnamiensis* LMG 10929 (44), *Burkholderia gladioli* 2-72 (58), *B. gladioli* 2-75 (58), *B. gladioli* 4-54 (58), *B. gladioli* 5-62 (58), *Burkholderia ubonensis* EY 3383 (73), *Burkholderia cocovenans* ATCC 33664, *Burkholderia pyrrhocina* ATCC 15958, *Burkholderia glathei* ATCC 29195, *Burkholderia caryophylli* Pe 102, *Burkholderia andropogonis* PA-133, *Burkholderia kururienensis* KP23 (74), *Burkholderia sacchari* IPT101 (6), *Burkholderia* sp. strain 2.2N (13), and *Burkholderia* sp. strain T-22-8A. *Ralstonia solanacearum* FC228, FC229, and FC230, *Pandora apista* LMG 16407 (19), *Pandora norimbergensis* LMG 18379 (19), *Pandora phenomena* LMG 18087 (19), *Pandora pulmonicola* LMG 18106 (19), *Stenotrophomonas maltophilia* XM16 (43), *S. maltophilia* XM47 (43), *Pseudomonas aeruginosa* PAO (38), *P. aeruginosa* PA14 (54), *Pseudomonas syringae* DC3000 (66), *Salmonella enterica* serovar Typhimurium SL1344 (37), *Serratia marcescens* H11, *Escherichia coli* TOP10 (Invitrogen), and *E. coli* S17-1 λ pir (59) were also used in this study. *E. coli* was grown at 37°C on Luria-Bertani (LB) agar (Lennox) or in LB broth (Lennox). *P. syringae*, *B. andropogonis*, *Burkholderia* sp. strain 2.2N, *Burkholderia* sp. strain T-22-8A, *B. glathei*, and *B. caryophylli* were grown at 25°C on LB agar or in LB broth containing 4% glycerol. All other bacterial strains were grown at 37°C on LB agar or in LB broth containing 4% glycerol. When appropriate, antibiotics were added at the following concentrations: 100 μ g of ampicillin per ml, 25 μ g of kanamycin per ml, and 15 μ g of tetracycline per ml for *E. coli*; and 100 μ g of streptomycin per ml and 50 μ g of tetracycline per ml for *B. pseudomallei* DD5025. In addition, *B. mallei* DD3008 was grown in the presence of 5 μ g of gentamicin per ml, and *B. mallei* NCTC 120(pBHR1-*wbiE*) was grown in the presence of 15 μ g of polymyxin B per ml and 5 μ g of kanamycin per ml.

MLST of *B. pseudomallei* 1026b. The pairs of primers used for PCR amplification and sequencing of the seven housekeeping gene fragments have been described previously (32). The multilocus sequence typing (MLST) database (www.mlst.net) identification number for *B. pseudomallei* 1026b is 208.

Subtractive hybridization. Subtractive hybridization was performed by using *B. pseudomallei* 1026b genomic DNA as the tester and *B. pseudomallei* K96243 genomic DNA as the driver. The protocol described in the CLONTECH PCR-Select bacterial genome subtraction kit user manual was followed, except that the hybridization temperature was 73°C instead of 63°C. The subtractive hybridiza-

tion products were cloned into pCR2.1-TOPO and transformed into chemically competent *E. coli* TOP10 cells.

Bacteriophage production, propagation, and DNA purification. The procedures used for bacteriophage production, propagation, and DNA purification have been described previously (70).

Enzyme-linked immunosorbent assay. The wells of a round-bottom microtiter plate were coated with approximately 5×10^6 bacteria in 100 μ l of 0.05 M carbonate buffer (pH 9.6), and the plate was incubated for 1 h at 37°C. The wells were washed with phosphate-buffered saline containing 0.05% Tween 20 and blocked with a 3% solution of skim milk in phosphate-buffered saline-Tween 20 for 1 h at 37°C. The wells were washed, a 1:1,000 dilution of monoclonal antibody 3D11 (Research Diagnostics, Inc.) was added, and the plate was incubated at 37°C for 1 h. Monoclonal antibody 3D11 is specific for the LPS O antigen of *B. mallei*. The wells were washed, and a 1:1,000 dilution of a peroxidase-labeled goat anti-mouse immunoglobulin G(H+L) [IgG(H+L)] antibody (KPL) was added to each well. The plate was incubated for 1 h at 37°C, washed, and developed with the 2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) (ABTS) peroxidase substrate system (KPL) for 10 min. The optical density at 410 nm was determined.

ϕ 1026b sensitivity testing. Approximately 10^2 PFU of ϕ 1026b was added to a saturated bacterial culture and incubated at 25°C for 20 min, and 4.8 ml of molten LB top agar (0.7%) containing 4% glycerol was added. The mixture was immediately poured onto an LB agar plate containing 4% glycerol and incubated overnight at 25 or 37°C, depending on the bacterial species being tested. Bacteria were considered to be sensitive to ϕ 1026b if they formed plaques under these conditions and resistant if they did not. The positive control, *B. mallei* ATCC 23344, formed plaques in the presence of ϕ 1026b after incubation at 25 and 37°C. No bacterial species tested formed plaques in the absence of ϕ 1026b.

Negative staining of ϕ 1026b. The procedure used for negatively staining bacteriophage ϕ 1026b with 1% phosphotungstic acid (PTA) (pH 6.6) has been described previously (70).

DNA manipulation and plasmid conjugation. Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals and were used according to the manufacturer's instructions. DNA fragments used in cloning procedures were excised from agarose gels and purified with a GeneClean III kit (Q · BIOgene). Bacterial genomic DNA was prepared by a previously described protocol (68). Plasmids were purified from overnight cultures by using Wizard Plus SV Minipreps (Promega). The suicide vector pDD94 was electroporated into *E. coli* S17-1 λ pir (12.25 kV/cm) and conjugated with *B. pseudomallei* 1026b for 8 h, as described elsewhere (23). The resulting strain, *B. pseudomallei* DD5025, contained pDD94 integrated into gene 59 of the ϕ 1026b prophage. Chromosomal DNA was isolated from DD5025 and digested with restriction endonuclease BamHI, and the bacteriophage attachment site and flanking bacterial DNA were obtained by self-cloning (23).

DNA sequencing and analysis. DNA sequencing was performed at ACGT, Inc. (Wheeling, Ill.) and at the LMT Sequencing Lab (Frederick, Md.). Most ϕ 1026b

TABLE 2. Subtractive hybridization products present in *B. pseudomallei* 1026b but not in *B. pseudomallei* K96243

Plasmid	Insert size (bp)	G+C content (%)	Protein function	Best BLASTP hit	E value	Accession no.
pSH3	756	53.6	Hypothetical protein	<i>Helicobacter pylori</i> J99	1e-04	NP_224110
				<i>Helicobacter pylori</i> 26695	3e-04	NP_208290
pSH4	235	55.7	Phage-related protein	Phage ϕ E125	7e-05	NP_536410
pSH5	1,113	57.8	Transposase B of insertion sequence ISBp1	<i>Burkholderia pseudomallei</i>	4e-77	AAG39072
pSH6	326	50.6	Putative Rossmann fold nucleotide-binding protein involved in DNA uptake	<i>Novosphingobium aromaticivorans</i>	2e-35	ZP_00093056
				<i>Thermoanaerobacter tengcongensis</i>	2e-14	NP_623068
pSH7	350	50.3	Putative ATP binding protein	<i>Ralstonia solanacearum</i>	2e-26	NP_521299
pSH8	321	52.0				
pSH10	1,217	47.6				
pSH11	517	52.2				
pSH13	432	44.2	Hypothetical protein	<i>Ralstonia metallidurans</i>	9e-64	ZP_00022203
				<i>Nostoc</i> sp. strain PCC 7120	3e-34	NP_487474
pSH18	1,440	49.2	Phage-related and hypothetical proteins	<i>Magnetococcus</i> sp. strain MC-1	3e-13	ZP_00042491
				<i>Nostoc punctiforme</i>	1e-42	ZP_00112321
				Phage ϕ E125		NP_536385
pSH19	478	48.9				
pSH21	379	43.5				
pSH23	270	53.7	Putative ATP binding protein	<i>Ralstonia solanacearum</i>	2e-13	NP_521299
pSH25	434	49.5	Phage-related integrase	<i>Xanthomonas axonopodis</i>	5e-05	NP_642500
				<i>Ralstonia metallidurans</i>	8e-05	ZP_00023697
pSH26	419	42.7				
pSH29	167	50.3				
pSH31	490	53.8	Putative acetyltransferase	<i>Methanosarcina mazei</i> Goe1	0.008	NP_632505
				<i>Pseudomonas aeruginosa</i> PAO1	0.019	NP_253367
pSH35	268	54.5				
pSH36	321	52.0	Putative site-specific DNA methyltransferase	<i>Cenarchaeum symbiosum</i>	5e-04	T31327
				<i>Pseudomonas syringae</i>	6e-04	ZP_00127033
pSH37	888	59.7	Phage-related and hypothetical proteins	Phage ϕ E125	3e-52	NP_536387
				<i>Bordetella parapertussis</i>	0.25	NP_884214
pSH38	741	46.8				
pSH40	441	50.3	Putative AraC family transcriptional regulator	<i>Vibrio vulnificus</i>	2e-06	NP_935090
				<i>Bradyrhizobium japonicum</i>	4e-06	NP_773722
pSH42	389	49.9	Putative ATP binding protein	<i>Ralstonia solanacearum</i>	1e-05	NP_521299
pSH47	790	45.1	Phage-related integrase	<i>Xanthomonas axonopodis</i>	7e-15	NP_643606
				<i>Vibrio parahaemolyticus</i>	3e-14	NP_797022
pSH49	235	55.7				
pSH51	531	46.1	Putative plasmid mobilization protein	<i>Zymomonas mobilis</i> plasmid ZM2	1.0	P15255
				<i>Treponema denticola</i> plasmid pTS1	2.9	NP_073756
				<i>Bartonella grahamii</i> plasmid pBGR1	3.4	NP_696963
pSH53	335	61.5				

genes were identified by using GeneMark.hmm (http://opal.biology.gatech.edu/GeneMark/gmhmm2_prok.cgi); other genes were identified by visual inspection, guided by BLAST (2) results. DNA and protein sequences were analyzed with GeneJockeyII and MacVector 7.2 software for the Macintosh computer. The gapped BLASTX and BLASTP programs were used to search the nonredundant sequence database for homologous proteins (2). The ϕ 1026b and ϕ E125 genomes were aligned by using BLAST 2 SEQUENCES (<http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/bl2.html>) with the Mega BLAST option selected.

Animal studies. Syrian hamsters (five animals per group) were infected intraperitoneally with 10^2 , 10^3 , and 10^4 1026b cells and 10^2 , 10^3 , and 10^4 DD5025 cells. The deaths in each group were monitored for 2 days, and the 50% lethal doses (LD_{50}) were determined. All of the animals died within 48 h of infection. This research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals* (<http://oacu.od.nih.gov/regs/guide/guidex.htm>). The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Phenotype microarray studies. PM1 and PM2 MicroPlates were purchased from BIOLOG (www.biolog.com) and were used according to the instructions supplied by the manufacturer.

Production of polyclonal antiserum against ϕ E125. One New Zealand rabbit was immunized with 1 ml of a 1:1 mixture of bacteriophage ϕ E125 ($\sim 10^5$ PFU) and the RIBI R-700 adjuvant system (Corixa). Five hundred microliters of the antigen-adjuvant mixture was injected intramuscularly into each hind leg on days

1 and 28. Antiserum was obtained by cardiac puncture on day 39 and was stored at -20°C until it was used.

Immunogold electron microscopy. The methods used for immunogold electron microscopy have been described previously (24). Briefly, ϕ E125 and ϕ 1026b were reacted with polyclonal rabbit antiserum directed against ϕ E125, washed, and reacted with goat anti-rabbit IgG gold conjugate (Sigma).

PCR amplifications. The sizes of PCR products were determined by agarose electrophoresis, and the products were cloned by using a pCR2.1 TOPO TA cloning kit (Invitrogen) and chemically competent *E. coli* TOP10 (Invitrogen). PCR amplifications were performed in 100- μ l (final volume) mixtures containing 1 \times Taq PCR master mix (QIAGEN), each oligodeoxyribonucleotide primer at a concentration of 1 μ M, and approximately 200 ng of genomic DNA. PCR mixtures were transferred to a PTC-150 MiniCycler with a Hot Bonnet accessory (MJ Research) and heated to 97°C for 5 min. This was followed by 30 cycles of a three-temperature cycling protocol (97°C for 30 s, 55°C for 30 s, and 72°C for 2 min) and one cycle at 72°C for 10 min. Genomic DNA from ϕ 1026b was used to PCR amplify an internal fragment of gene 59 with the following oligodeoxyribonucleotide primer pair: MFS-2 (5'-ACAACCTGTCTCTGTTGCTG-3') and MFS-3 (5'-CTGGAACATGTCGCTAAGC-3').

In order to determine the order and orientation of the HindIII fragments in the intact ϕ 1026b genome, outward-oriented primers specific for the ends of each HindIII fragment (except the 126-, 602-, and 1,068-bp fragments) were synthesized, and PCRs were performed with ϕ 1026b genomic DNA and all possible primer combinations. It was hypothesized that two HindIII fragments were adjacent if a PCR product was obtained with primer pairs specific for the

TABLE 3. Bacteriophage ϕ 1026b plaque formation on *B. mallei* strains

Strain	Relevant characteristics	Plaque formation	Reference or source
NCTC 120	LPS O-antigen mutant; <i>wbiE</i> ::IS407A	—	70
NCTC 120 (pBHR1- <i>wbiE</i>)	LPS O antigen positive; <i>wbiE</i> provided in trans on pBHR1	+	70
NCTC 10248		+	70
NCTC 10229		+	70
NCTC 10260		+	70
NCTC 10247		+	70
NCTC 3708		+	70
NCTC 3709		+	70
ATCC 23344	Type strain; genomic sequence completed ^a	+	70
ATCC 10399		+	70
ATCC 15310	Produces LPS O antigen	+	70
DB110795	Laboratory-passaged ATCC 15310; LPS O-antigen mutant; <i>wbiG</i> ::IS407A	—	70
BML1	Lysogen; ATCC 23344 (ϕ 1026b)	—	This study
BML10	Lysogen; ATCC 23344 (ϕ E125)	—	70
DD3008	ATCC 23344 derivative; capsule mutant	+	27
2002721273		+	30
2002721274		+	30
2002721276		+	30
2002721277		+	30
2002721278		+	30
2002721279		+	30
2002721280		+	30
2000031064		+	30
2000031065		+	30
2000031066		+	30
Turkey 1		+	USDA ^b
Turkey 2		+	USDA
Turkey 3		+	USDA
Turkey 4	Produces LPS O antigen	—	USDA
Turkey 5	Produces LPS O antigen	—	USDA
Turkey 6		+	USDA
Turkey 7		+	USDA
Turkey 8		+	USDA
Turkey 9		+	USDA
Turkey 10		+	USDA
ISU	LPS O-antigen mutant; genetic mutation unknown	—	USDA

^a <http://www.tigr.org/>.^b USDA, United States Department of Agriculture.

corresponding ends of those fragments. All PCR products were cloned and sequenced to confirm the PCR results. The sequences of the 16 oligodeoxynucleotide primers used in this analysis were as follows: 8.4R, 5'-GTGCTGTC GCACTAATCATG-3'; 3.6L, 5'-CAACGGAAGAGTCGCGATTG-3'; 3.6R, 5'-CCGACGATCTGATCAAGATC-3'; 3.1L, 5'-TGCTGCTGAAACGATATT GC-3'; 3.1R, 5'-ATCGTGAAACTCGGCGTGTC-3'; 12.8L, 5'-AACGCGCTT TGTCGATCGTG-3'; 12.8R, 5'-ACCATCTCGAAGAGTTCTGTC-3'; 9.3L, 5'-TCAAGGTAGAACAGCGTGTC-3'; 9.3R, 5'-CAGCGCTCACGTAGTTCA AG-3'; 9.8A, 5'-TCTGACAATTCGATACGCGTG-3'; 9.6B, 5'-AAGCTCGAG ACGTTTCTTG-3'; 2.5L-2, 5'-TAGCCACTCGCGAAACATCG-3'; 2.5R, 5'-TGGTTTATCGTTGCGCATG-3'; 3.8L, 5'-GCCCTTACTTTCATTGAAC C-3'; 3.8R, 5'-AAGAGGACTCGCCGATCAAC-3'; and 8.4L, 5'-ATCGCAGT TCGCCATGCAAC-3'.

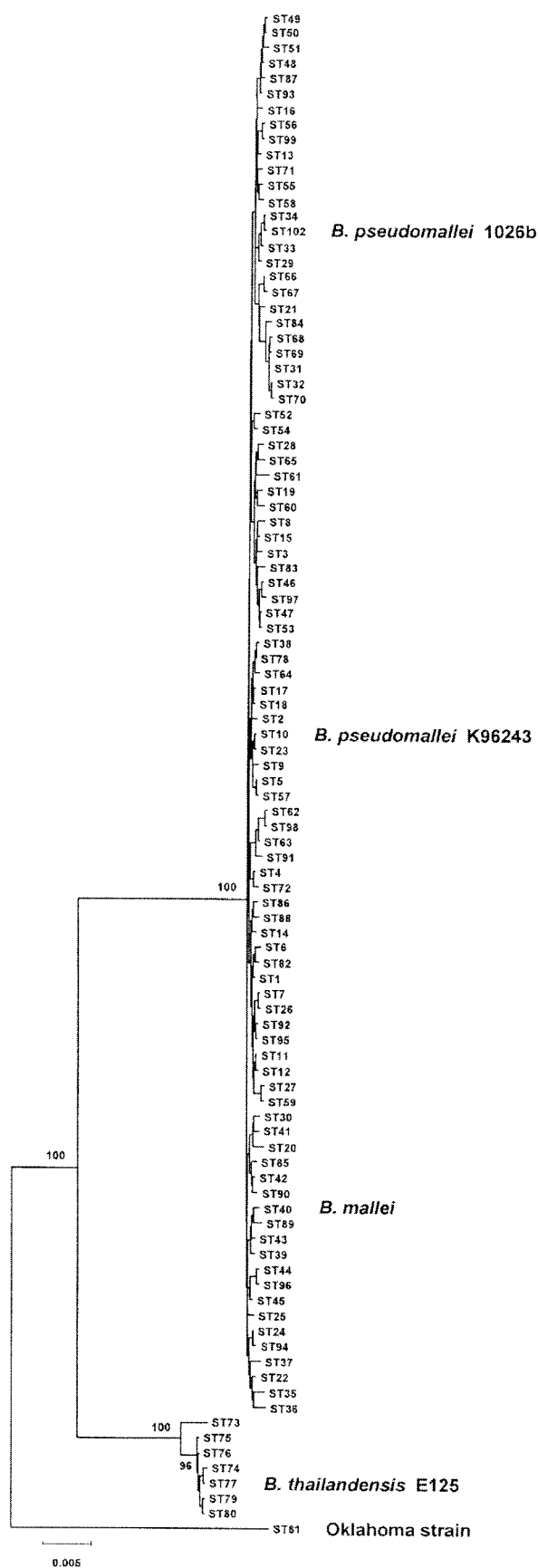
PCRs were performed with genomic DNAs from *B. pseudomallei* K96243 and 1026b, *B. mallei* ATCC 23344 and BML1, and ϕ 1026b and with primers Int2 (5'-CACCAGACGAGAAGATGACTG-3') and Int5 (5'-TTGAATCGCACCGT TTGGTG-3') to determine if ϕ 1026b integrated into the *tRNA*^{Pro-3} gene. A single PCR product of the expected size (447 bp) was obtained with *B. mallei* BML1 DNA and *B. pseudomallei* 1026b DNA. This product was cloned, and its nucleotide sequence was determined. As expected, no PCR products were obtained when genomic DNAs from *B. pseudomallei* K96243, *B. mallei* ATCC 23344, and ϕ 1026b were used in the PCR.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in the GenBank database under accession numbers AY471580 to AY471606 (1026b-K96243 subtractive hybridization products) and AY453853 (bacteriophage ϕ 1026b).

RESULTS

MLST of *B. pseudomallei* 1026b. Godoy et al. developed an MLST scheme for *B. pseudomallei*, *B. mallei*, and *B. thailandensis* based on sequence variations in seven housekeeping genes, but *B. pseudomallei* 1026b was not one of the isolates examined (32). In this study, the allelic profile of 1026b was determined to be 3-4-12-1-1-4-1, which corresponds to a new sequence type (ST102). Figure 1 shows a minimum-evolution tree based on the concatenated sequences of the seven MLST loci for 92 sequence types of *B. pseudomallei*. Note that *B. mallei* isolates (ST40) cluster with *B. pseudomallei* isolates on the minimum-evolution tree and are considered to be a distinct clone of *B. pseudomallei* (32). Clinical isolates 1026b and K96243 were resolved into two genetically distinct clones, ST102 and ST10, based on the MLST analysis (Fig. 1). The MLST results suggest that 1026b and K96243 are excellent candidates for examining the genomic diversity of *B. pseudomallei*.

***B. pseudomallei* 1026b-specific subtractive hybridization library contains multiple mobile genetic elements.** The goal of



this study was to identify genetic determinants present in *B. pseudomallei* 1026b but not in *B. pseudomallei* K96243 by subtractive hybridization. Forty plasmid inserts from a 1026b-K96243 subtractive hybridization library were identified and used to perform BLASTN searches with the completed K96243 genome (<http://www.sanger.ac.uk/>). Twenty-seven of the subtractive hybridization products were not present in K96243 (Table 2). The sizes of the 1026b-specific subtractive hybridization products ranged from 167 to 1,440 bp, and these products had relatively low G+C contents compared to the G+C content of the K96243 genome (68.1%). The putative functions of proteins encoded by genes in the subtractive hybridization library included several mobile genetic elements (51), including phage-related proteins, an insertion sequence element, and a plasmid-like mobilization protein (Table 2). Plasmids pSH4, pSH18, pSH25, pSH37, and pSH47 encode phage-related proteins. These proteins include phage-related integrases (pSH25 and pSH47) and several proteins that are similar to bacteriophage ϕ E125 proteins (pSH4, pSH18, and pSH37). The IS3 family insertion sequence *ISBp1* (69) was present in plasmid pSH5 (Table 2). Woo et al. previously demonstrated that *ISBp1* was present in ~65% of *B. pseudomallei* strains but was absent from K96243 (69). The nucleotide sequence of the pSH5 DNA insert suggests that *ISBp1* is also present in the genome of *B. pseudomallei* 1026b. The 531-bp subtractive hybridization insert of plasmid pSH51 encodes a putative plasmid mobilization protein that was also isolated from a *B. pseudomallei* 1026b-*B. thailandensis* E264 subtractive library (55). No plasmids have been described in *B. pseudomallei* 1026b, and the pSH51 insert may represent an integrated plasmid that is not present in *B. pseudomallei* K96243 or *B. thailandensis* E264. Three plasmid inserts, pSH7, pSH23, and pSH42, encoded distinct regions of a putative ATP binding protein (Table 2). Finally, 13 subtractive hybridization products encoded hypothetical proteins (pSH3 and pSH13) or novel proteins (pSH8, pSH10, pSH11, pSH19, pSH21, pSH26, pSH29, pSH35, pSH38, pSH49, and pSH53) (Table 2). Taken together, the results demonstrate the genomic diversity of these two clinical isolates of *B. pseudomallei*, especially with respect to mobile genetic elements and novel gene sequences.

***B. pseudomallei* 1026b spontaneously produces a bacteriophage that is specific for *B. mallei*.** The subtractive hybridization product library contained multiple 1026b-specific bacteriophage sequences, and it was of interest to see if this strain actually produced a bacteriophage. *B. mallei* was chosen as a host because previous studies demonstrated that it is suscep-

FIG. 1. Minimum-evolution tree constructed from the concatenated sequences of seven MLST loci. The seven housekeeping genes used for the MLST scheme are *ace*, *glbB*, *gmhD*, *lepA*, *lipA*, *narK*, and *ndh* (32). The concatenated sequences from 100 sequence types, representing isolates of *B. pseudomallei*, *B. mallei*, and *B. thailandensis* and the Oklahoma strain (72), were used to construct the minimum-evolution tree. The positions of the *B. mallei* clone (ST40), *B. pseudomallei* 1026b (ST102), *B. pseudomallei* K96243 (ST10), *B. thailandensis* E125 (ST77), and the Oklahoma strain (ST81) are indicated. The levels of recovery of the major nodes in 1,000 bootstrap replicates (expressed as percentages) are also indicated. Bar = differences at 0.5% of the nucleotide sites.

tible to infection with *B. pseudomallei* and *B. thailandensis* bacteriophages (45, 62, 70). ϕ 1026b spontaneously produced a bacteriophage, designated ϕ 1026b, that formed turbid plaques with a diameter of 1.5 to 2.0 mm on *B. mallei* ATCC 23344. No other plaque types were identified, which suggests that 1026b produces only one bacteriophage under the growth conditions used. However, it is possible that 1026b produces additional bacteriophages that cannot use *B. mallei* as a host. Bacteriophage production was only slightly increased by brief exposure to UV light (470 versus 540 PFU/ml). After infection, the ϕ 1026b genome integrated into the *B. mallei* chromosome at a specific site and became a prophage (see below). *B. mallei* ATCC 23344 was infected with ϕ 1026b, and a lysogenic derivative was isolated and designated BML1 (Table 3).

Bacteriophage ϕ 1026b formed plaques on 29 of 36 *B. mallei* strains used in this study (Table 3). Bacteriophages initiate infection by specifically binding to a surface receptor on the bacterial host, such as LPS O antigen and capsular polysaccharide. LPS O-antigen production by *B. mallei* strains was examined by an enzyme-linked immunosorbent assay by using monoclonal antibody 3D11 (Table 3). Three of the ϕ 1026b-resistant *B. mallei* strains, NCTC 120, DB110795, and ISU, did not produce LPS O antigen. When LPS O-antigen production in *B. mallei* NCTC 120 was complemented by providing pBHR1-*wbiE* in *trans*, the resulting strain was susceptible to infection with bacteriophage ϕ 1026b (Table 3). Surprisingly, *B. mallei* strains Turkey 4 and Turkey 5 were resistant to infection with ϕ 1026b even though they produced LPS O antigen (Table 3). The *B. mallei* lysogens BML1 and BML10 produced LPS O antigen and were resistant to infection with ϕ 1026b, presumably due to immunity or superinfection exclusion proteins encoded by the prophages that they harbor. Capsular polysaccharide was not required for plaque formation as ϕ 1026b formed plaques on DD3008, a capsule-deficient mutant derived from ATCC 23344 (Table 3). The host range of ϕ 1026b was further examined by using *B. pseudomallei*, *B. thailandensis*, *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. gladioli*, *B. uboniae*, *B. cocovenans*, *B. pyrrocinia*, *B. glathei*, *B. caryophylli*, *B. andropogonis*, *B. kururiensis*, *Burkholderia* sp. strain 2.2N, *Burkholderia* sp. strain T-22-8A, *P. apista*, *P. norimbergensis*, *P. pnompenusa*, *P. pulmonicola*, *P. aeruginosa*, *P. syringae*, *R. solanacearum*, *S. maltophilia*, *S. enterica* serovar Typhimurium, *S. marcescens*, and *E. coli*. Bacteriophage ϕ 1026b formed plaques with none of these bacteria. These results demonstrate that bacteriophage ϕ 1026b forms plaques only on *B. mallei* strains and that LPS O antigen is required but is not sufficient for plaque formation by ϕ 1026b. Note that the host range of ϕ 1026b is identical to the host range of bacteriophage ϕ E125 (70). These results suggest that bacteriophage ϕ 1026b may be a useful diagnostic tool for differentiating *B. pseudomallei* and *B. mallei*, two closely related biological threat agents (56). However, there is no advantage to using ϕ 1026b rather than ϕ E125 for this application (70).

Bacteriophage ϕ 1026b has an isometric head and a long, noncontractile tail. Bacteriophages may be tailed, polyhedral, filamentous, or pleomorphic and can be classified by morphology and by the nature of the nucleic acid (1). Numerous negatively stained bacteriophages were examined, and a representative image of ϕ 1026b is shown in Fig. 2. ϕ 1026b possessed an isometric head that was 56 nm in diameter and a

long, noncontractile tail that was approximately 200 nm long and 8 nm in diameter. Based on its morphotype, ϕ 1026b can be classified as a member of the order *Caudovirales* and the family *Siphoviridae* (1).

Molecular characterization of the bacteriophage ϕ 1026b genome. The ϕ 1026b genome was digested with HindIII, and 11 fragments were generated; these fragments were 0.1, 0.6, 1.1, 2.5, 3.1, 3.6, 3.8, 8.4, 9.4, 9.5, and 12.7 kb long. The fragments were heated to 80°C, and the 8.4-kb fragment dissociated into two fragments (2.3 and 6.1 kb), suggesting that a cohesive (*cos*) site was present (data not shown). The 11 HindIII fragments were cloned, and their nucleotide sequences were determined. The nucleotide sequencing results are shown schematically in Fig. 3. The subtractive hybridization DNA insert of pSH4 was identical to bacteriophage ϕ 1026b from position 44417 to position 44651, which included the 3' ends of gene 63 and gene 64 (Table 2 and Fig. 3).

The ϕ 1026b genome is a linear molecule that is 54,865 bp long, and it contains 10-base 3' single-stranded extensions on the left (3'-GCGGGCGAAG-5') and right (5'-CGCCCGCTT C-3'), as shown in Fig. 3. The *cos* site of ϕ 1026b is identical to the *cos* site of bacteriophage ϕ E125 (70). The G+C content of the ϕ 1026b genome is 60.7%, which is lower than the G+C content of the *B. pseudomallei* K96243 genome (68.1%) (<http://www.sanger.ac.uk/>). The ϕ 1026b genome encodes 83 proteins, and 58 of these proteins generated best hits to bacteriophage ϕ E125 proteins when the BLASTP search algorithm was used.

Bacteriophage genomes are composed of a mosaic of multigene modules, each of which encodes a group of proteins involved in a common function, such as DNA packaging, head biosynthesis, tail biosynthesis, host lysis, lysogeny, or replication (10, 36, 40). The ϕ 1026b genome contains multigene modules involved in DNA packaging, head morphogenesis, tail morphogenesis, host lysis, and DNA replication (Fig. 3). The relative order of these modules in the ϕ 1026b genome is similar to the order in other *Siphoviridae* genomes (10, 40, 70). ϕ 1026b also encodes a LysR family transcriptional regulator (57) and a major facilitator superfamily (MFS) transporter (52), encoded by gene 58 and gene 59 (Fig. 3). It is interesting that these genes have been found in tandem in several recently completed bacterial genomes, including those of *R. solanacearum*, *B. fungorum*, and *P. syringae*. gp59 is a member of the metabolite:H⁺ symporter family of MFS proteins which function by proton symport and allow the uptake of a wide variety of metabolites (52).

Temperate bacteriophage genomes often contain an attachment site (*attP*) utilized for integration into a homologous region within the bacterial genome (*attB*) via site-specific recombination (20). The *attP* site of ϕ 1026b was adjacent to the site encoding gp33, a site-specific integrase (Fig. 3). The nucleotide sequence of *attP* contained a 49-bp sequence that was identical to *attB* sequences present in the genomes of *B. mallei* ATCC 23344 and *B. pseudomallei* K96243. This sequence corresponded to the 3' end of the tRNA^{Pro-3} gene on chromosome 1 of *B. mallei* (positions 830691 to 830615) and chromosome 1 of *B. pseudomallei* (positions 1604091 to 1604043). tRNA genes often serve as target sequences for site-specific integration of temperate bacteriophages, plasmids, and pathogenicity islands (14, 34). The *attP* site of ϕ 1026b was identical

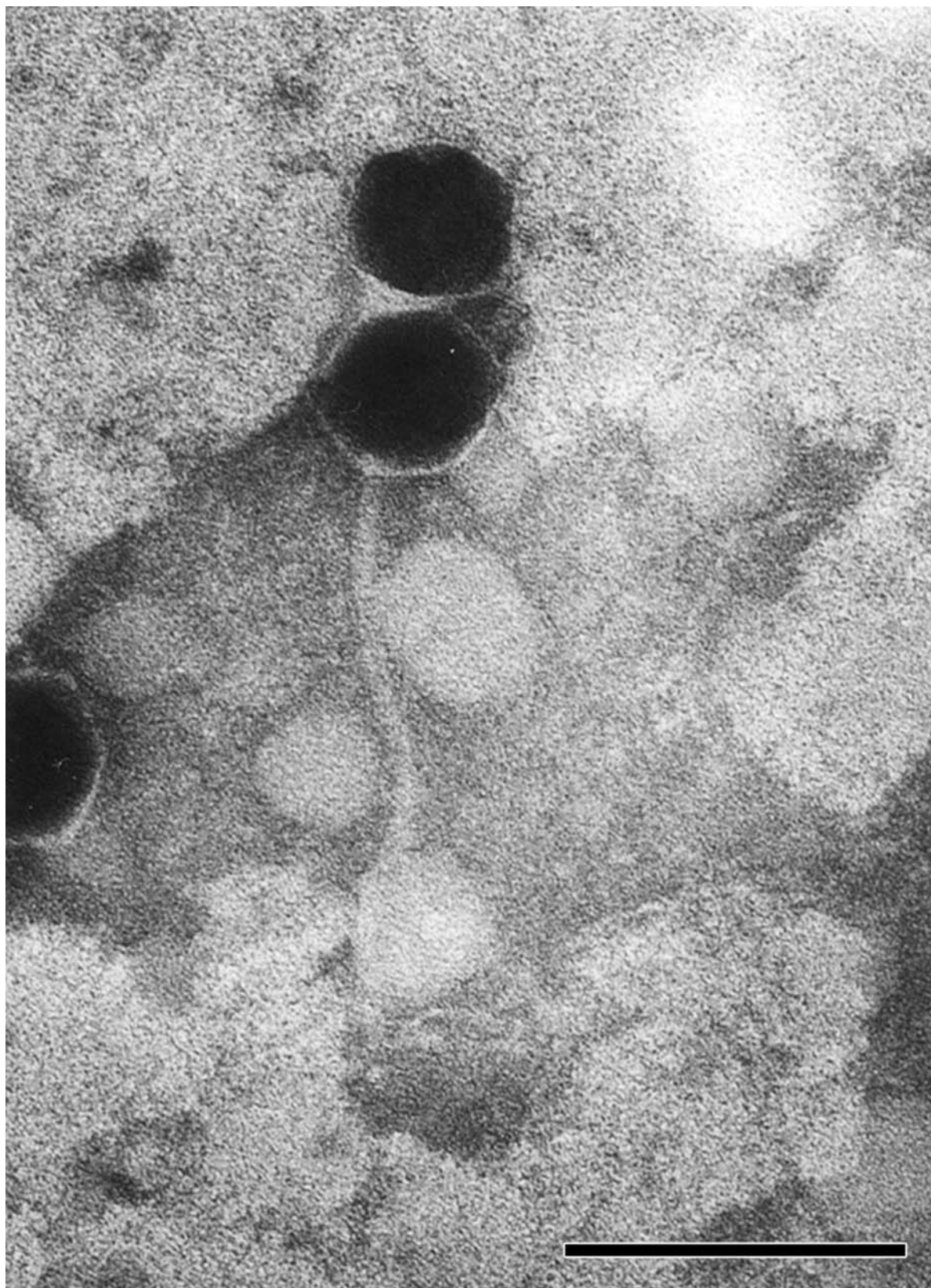


FIG. 2. Transmission electron micrograph of bacteriophage $\phi 1026b$ negatively stained with 1% PTA. One intact bacteriophage (head and tail) and one bacteriophage head without an attached tail are shown. Scale bar = 100 nm.

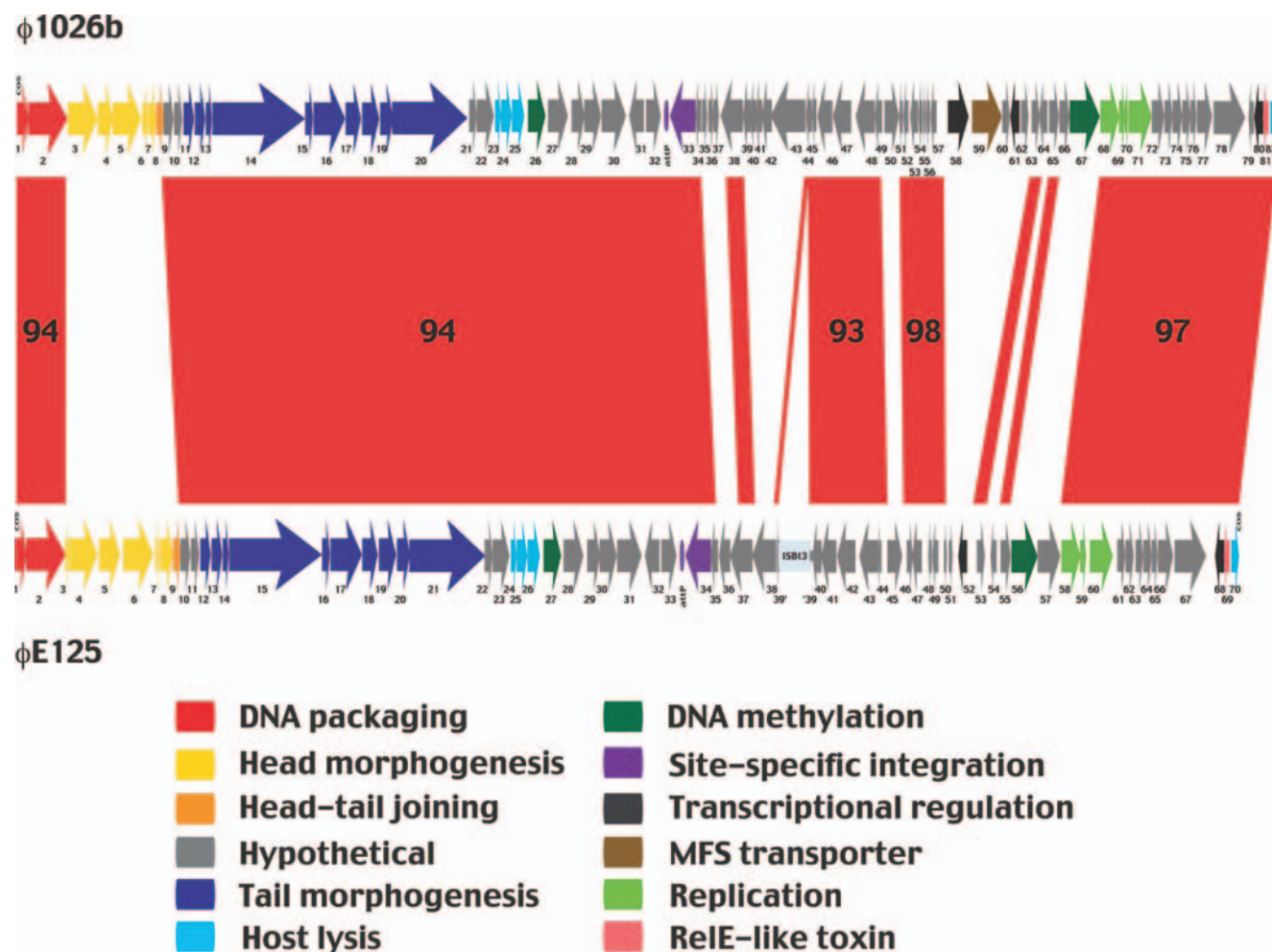


FIG. 3. Comparative analysis of the genomes of *Burkholderia* bacteriophages ϕ 1026b and ϕ E125. The genomes of temperate bacteriophages ϕ 1026b and ϕ E125 are depicted schematically at the top and bottom, respectively. Red indicates DNA sequences that are present in both bacteriophages, and the numbers in the red areas indicate the percentages of nucleotide identity in conserved regions that are 1 kb long or longer. The putative functions of proteins encoded by ϕ 1026b genes are color coded, and insertion sequence *ISBt3* in gene 39 of ϕ E125 is indicated by pale blue. Gene 25a (ϕ 1026b) and gene 26a (ϕ E125) are not shown for clarity.

to the *attP* site of ϕ E125 (70). It is worth emphasizing that *B. pseudomallei* 1026b and *B. thailandensis* E125 both contain bacteriophages integrated at *tRNA*^{Pro-3}, while *B. pseudomallei* K96243 does not (Fig. 1). However, *B. pseudomallei* K96243 does have a prophage-like region on chromosome 2 that is 98% identical to ϕ 1026b gene 48 to gene 52 and 97% identical to ϕ 1026b gene 55 to gene 57 (<http://www.sanger.ac.uk/>). It is not known if this is a functional or defective prophage.

Comparative analysis of the genomes of temperate bacteriophages ϕ 1026b and ϕ E125. The host range and morphology of ϕ 1026b are remarkably similar to the host range and morphology of ϕ E125 (70), a temperate bacteriophage harbored by *B. thailandensis* E125 (Fig. 1). As mentioned above, the two genomes contain identical *cos* and *attP* sites, and 70% of the ϕ 1026b proteins generate best hits to ϕ E125 proteins when the BLASTP search algorithm is used. The genome of ϕ 1026b is marginally larger (1.5 kb) than the genome of ϕ E125. Figure 3 shows a comparative analysis of the genomes of ϕ 1026b and ϕ E125 generated by using the BLAST 2 SEQUENCES program (63). Large segments of DNA are shared by the two

genomes, and the levels of nucleotide identity are 93 to 98%. These conserved regions are interspersed with DNA segments that exhibit little or no sequence similarity (Fig. 3). The mosaic nature of the genomes is illustrated by the head morphogenesis and head-tail joining genes in ϕ 1026b (gene 3 to gene 8) and ϕ E125 (gene 3 to gene 9). The ϕ 1026b genes more closely resemble head morphogenesis and head-tail joining genes of *P. aeruginosa* and *Pseudomonas putida* bacteriophages than the corresponding genes in ϕ E125. However, the DNA packaging and tail morphogenesis genes flanking this region in ϕ 1026b and ϕ E125 are 94% identical (Fig. 3). The most likely explanation for this finding is that recombination between one of these bacteriophages and an unrelated bacteriophage (or prophage) resulted in acquisition of a different set of head morphogenesis and head-tail joining genes (11, 36). Because the proteins involved in head morphogenesis interact with one another, it is not surprising that the genes encoding them are laterally acquired as a group. The putative crossover points for this recombination event and those described below occur at or near gene boundaries. The modular exchange of head mor-

phogenesis genes suggests that DNA packaging proteins (gp1 and gp2) can associate with two distinct head protein sets, while head-to-tail association seems to require the mediation of a specific head-tail joining protein (gp8 in ϕ 1026b and gp9 in ϕ E125).

Genetic mosaicism was readily evident in the region spanning the site-specific integrase and DNA replication genes of ϕ 1026b and ϕ E125 (Fig. 3). This large mosaic region includes five modules of conserved genes and six modules of genes with no sequence similarity. Note that one of the conserved modules in ϕ E125 is disrupted by an *ISBt3* insertion in gene 39 (70), which corresponds to gene 44 in ϕ 1026b (Fig. 3). The biological function(s) of this large mosaic region probably includes lysogeny, lysogenic conversion, and superinfection immunity (10, 39). As mentioned above, bacteriophage ϕ 1026b cannot form plaques on the lysogens BML1 and BML10 (Table 3). In comparison, bacteriophage ϕ E125 can form plaques on BML1 but not on BML10. This indicates that the ϕ E125 lysogen (BML10) can prevent superinfection with both ϕ E125 and ϕ 1026b but that the ϕ 1026b lysogen (BML1) can prevent superinfection only with ϕ 1026b. It seems likely that one or more of the novel gene modules in the mosaic region are responsible for the differences in superinfection immunity, but further studies are required to prove this.

Several additional features of the large mosaic region should be mentioned here. First, ϕ 1026b gene 66 and gene 67 were replaced in ϕ E125 by gene 56 and gene 57 (Fig. 3). This modular replacement occurred precisely at the gene boundaries, suggesting that these gene pairs perform analogous functions. The biological function likely involves DNA methylation because both gene 67 (ϕ 1026b) and gene 56 (ϕ E125) encode DNA methyltransferases. Interestingly, DNA methyltransferases are relatively common in bacteriophages from gram-positive bacteria but not in bacteriophages from gram-negative bacteria. Second, single-gene modular replacement between ϕ 1026b gene 50 and ϕ E125 gene 45 also occurred, but the biological importance of this exchange is not known because it involved genes with no known functions (Fig. 3). Finally, the large mosaic region of ϕ 1026b includes gene 58 and gene 59, genes that encode a LysR family transcriptional regulator and an MFS transporter (Fig. 3). These genes were not present in the ϕ E125 genome, supporting the notion that they were acquired by lateral gene transfer from a bacterial genome (see above).

Phenotypic analysis of *B. pseudomallei* DD5025. The prophage-encoded MFS transporter (gp59) may provide *B. pseudomallei* 1026b with a selective advantage over other *B. pseudomallei* strains by allowing the uptake of a nutrient(s) from the environment (36, 52). In order to examine the function of gene 59, a strain harboring a mutation in this gene was constructed. An internal gene fragment of gene 59 was PCR amplified and cloned into the suicide vector pSKM11 (Table 1). Plasmid pDD94 was mobilized into *B. pseudomallei* 1026b, and the resulting merodiploid strain was designated DD5025. There were no detectable differences between the growth of 1026b and the growth of DD5025 in complex or defined media (data not shown). Both strains were examined to determine their abilities to metabolize 190 different carbon sources by using PM1 and PM2 phenotype microarrays (www.biolog.com), but no differences were observed. Prophage-encoded

virulence factors in other bacterial species have been described (5), and it was of interest to see if gene 59 provided a selective benefit to 1026b in an animal model of melioidosis (25). Syrian hamsters were infected intraperitoneally with 10^2 , 10^3 , and 10^4 cells of 1026b and DD5025, and the LD₅₀s were determined 2 days postinfection. The LD₅₀ for both strains was $<10^2$ cells, suggesting that gene 59 is not important for the pathogenesis of 1026b in this animal model of melioidosis.

Immunogold electron microscopy of ϕ E125 and ϕ 1026b.

The comparative genomics analysis of ϕ E125 and ϕ 1026b predicted that these phages contain antigenically related tails but antigenically distinct heads (Fig. 3). Immunogold electron microscopy was performed to see if polyclonal antiserum against ϕ E125 reacted with ϕ 1026b (Fig. 4). The bacteriophages were reacted with polyclonal rabbit antiserum directed against ϕ E125, washed, and reacted with a goat anti-rabbit IgG gold conjugate. As expected, the antibodies reacted with the head and tail of bacteriophage ϕ E125 (Fig. 4). The anti- ϕ E125 antibodies did not react with the head of ϕ 1026b but did react with the tail (Fig. 4). These results corroborate the comparative genomics results and demonstrate that the tails of bacteriophages ϕ E125 and ϕ 1026b are antigenically related but the heads are antigenically distinct. Tailed bacteriophages bind to the surfaces of their bacterial hosts by using their tails, and the genetic and antigenic relatedness of the tails of ϕ E125 and ϕ 1026b probably accounts for their specificity for *B. mallei*.

DISCUSSION

The results presented here demonstrate that clinical isolates of *B. pseudomallei* exhibit genetic diversity, especially with regard to the mobile genetic elements that they harbor. It should be emphasized that only two clinical strains were compared in this study, but it is likely that future *B. pseudomallei* genome sequencing and comparative genome hybridization projects will yield similar results. At least five prophages (or prophage-like elements) were identified in *B. pseudomallei* 1026b but not in *B. pseudomallei* K96243 (<http://www.sanger.ac.uk/>). Prophages are a major source of strain-specific differences in several pathogenic bacteria, including Shiga toxin-producing *E. coli*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *S. enterica*, and *Xylella fastidiosa* (11, 16, 17). The genomic sequencing results for multiple strains of *Streptococcus* and *Xylella* suggest that different disease pathologies may be due to differences in the prophage contents of the infecting strains (48, 64). Prophages are responsible for much of the laterally transferred DNA in bacteria, and they play a major role in the evolution of bacterial pathogens by providing new virulence determinants (5, 15). Manzeniuk et al. found that 92% of *B. pseudomallei* strains produced temperate bacteriophages, demonstrating that prophages are relatively common in this bacterial species (45). Brown and Beacham performed subtractive hybridization between *B. pseudomallei* and *B. thailandensis* and identified multiple *B. pseudomallei*-specific mobile genetic elements, including a P2-like prophage (9). Taken together, the results demonstrate that there is considerable diversity in the mobile genetic elements that *B. pseudomallei* strains harbor. It is tempting to speculate that the variable clinical presentation of melioidosis is due, at least in part, to the prophage contents of the infecting *B. pseudomallei* strains. The genomic sequences

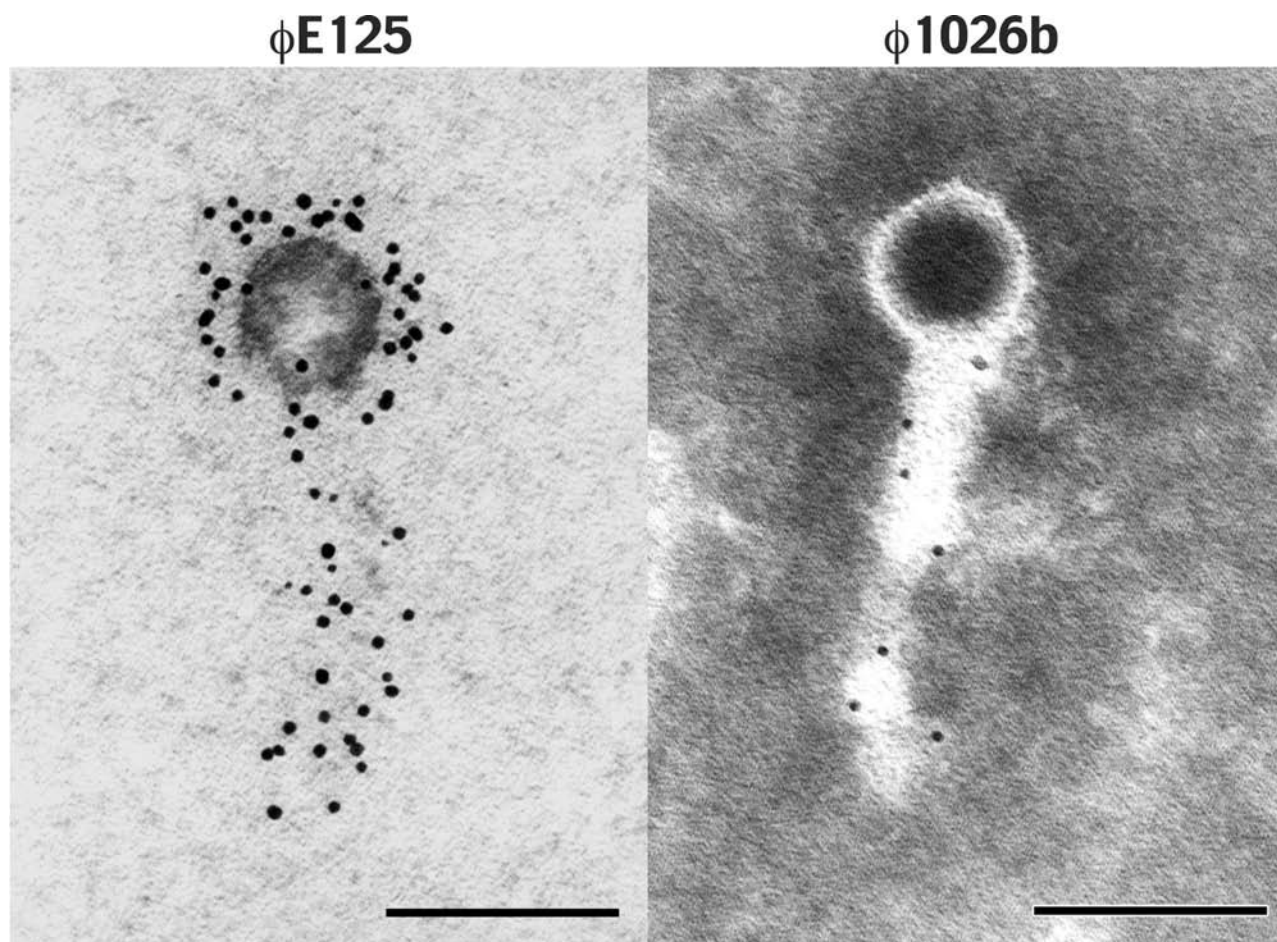


FIG. 4. Immunogold electron microscopy of bacteriophages ϕ E125 and ϕ 1026b. The bacteriophages were reacted with polyclonal rabbit antiserum directed against ϕ E125, washed, and reacted with goat anti-rabbit IgG gold conjugate (5 nm). Bacteriophage ϕ 1026b was subsequently negatively stained with 1% PTA. Scale bar = 100 nm.

of additional *B. pseudomallei* strains isolated from melioidosis patients with defined clinical manifestations are needed to further explore this possibility.

It is widely accepted that tailed bacteriophage genomes are a mosaic collection of genetic material resulting from recombination between bacteriophages (or prophages) (11, 16, 17, 36). A comparative genome analysis of ϕ 1026b and ϕ E125 revealed regions with high sequence similarity interspersed with regions displaying no sequence similarity (Fig. 3). This mosaic genetic relationship indicates that recombination between ϕ 1026b or ϕ E125 and an unrelated bacteriophage(s) occurred during the evolution of these *Burkholderia* bacteriophages, which resulted in acquisition of new head and lysogeny genes. The ϕ 1026b head morphogenesis genes more closely resemble the head morphogenesis genes of *P. aeruginosa* and *P. putida* bacteriophages than the corresponding genes in ϕ E125. In addition, the host lysis cassettes of ϕ 1026b (genes 23 to 25) and ϕ E125 (genes 24 to 26) are located directly downstream of the putative tail fiber module, which is similar to the genetic organization of *P. aeruginosa* bacteriophage D3 (42). This genetic organization is commonly found in *Siphoviridae* from low-G+C-content gram-positive bacteria (10) but not in *Siphoviridae* from gram-negative bacteria. The tail fiber mod-

ule-host lysis cassette module organization seems to be an ancestral trait in at least a subgroup of *Burkholderia* and *Pseudomonas Siphoviridae*.

It is curious that bacteriophages ϕ 1026b and ϕ E125 specifically infect *B. mallei* but are harbored by *B. pseudomallei* and *B. thailandensis*. What is the mechanism by which *B. pseudomallei* and *B. thailandensis* strains are resistant to infections with ϕ 1026b and ϕ E125? First, *B. pseudomallei* and *B. thailandensis* strains may be immune to superinfection with these bacteriophages because they harbor similar prophages. The genomic sequence of *B. pseudomallei* K96243 contains eight genes that are nearly identical to ϕ 1026b genes (<http://www.sanger.ac.uk/>), and Woods et al. (70) found that 31% of *B. thailandensis* strains harbor a ϕ E125-like prophage. Thus, it is clear that some *B. pseudomallei* and *B. thailandensis* strains are lysogenic and may be immune to superinfection with ϕ 1026b and ϕ E125. However, 69% of *B. thailandensis* strains did not possess an ϕ E125-like prophage, suggesting that superinfection immunity alone is not responsible for their resistance to infection with ϕ E125 (70). Second, there may be differences in the bacteriophage receptors present on *B. mallei* and on *B. pseudomallei* and *B. thailandensis*. LPS O antigen is required for plaque formation on *B. mallei*, indicating that this is the

surface-exposed bacteriophage receptor (Table 3). *B. mallei* LPS O antigen is similar to the antigen previously described for *B. pseudomallei* and *B. thailandensis* except that it is devoid of an O-acetyl group at the 4' position of the L-talose residue (7, 12, 41, 53). *B. pseudomallei* and *B. thailandensis* strains may be resistant to infection with ϕ 1026b and ϕ E125 because the O-acetyl group at the 4' position of the L-talose residue alters the conformation of the LPS O antigen and/or blocks the bacteriophage binding site. Finally, *B. pseudomallei* and *B. thailandensis* may be resistant to infection with these bacteriophages because they do not produce a coreceptor. ϕ 1026b and ϕ E125 do not form plaques on *B. mallei* strains Turkey 4 and Turkey 5, two strains that produce LPS O antigen (Table 3). Taken together, the results indicate that LPS O antigen is required, but is not sufficient, for infection with these bacteriophages. It is possible that *B. mallei* strains Turkey 4 and Turkey 5 do not produce a coreceptor that participates with LPS O antigen in the initial interaction with ϕ 1026b and ϕ E125. Further studies are required to identify and characterize this putative coreceptor and examine if it is present in *B. pseudomallei* and *B. thailandensis*.

ϕ 1026b gene 58 and gene 59 encode a LysR family transcriptional regulator (57) and an MFS transporter (52), respectively. These genes are not present in the ϕ E125 genome, but similar gene pairs are present in several bacterial genomes (Fig. 3). Given this information, it is feasible that ϕ 1026b gene 58 and gene 59 were acquired together by lateral transfer from a bacterial genome. The tandem arrangement of these genes in diverse genomes suggests that they may function together. One obvious possibility is that expression of the MFS transporter is regulated by the LysR family transcriptional regulator. It is hypothesized that gene 58 and gene 59 provide a selective advantage to *B. pseudomallei* 1026b by allowing it to take up a solute(s) from the environment that may not be accessible to other bacteria, including other strains of *B. pseudomallei*. In addition, the genes may also benefit the prophage by ensuring that it is maintained in the chromosome of its host. Unfortunately, no phenotype was observed for a strain (DD5025) harboring a mutation in gene 59. There was no difference in the growth, virulence, or catabolism of 190 carbon sources between 1026b and DD5025. Preliminary studies have indicated that while these two strains have similar growth rates in brain heart infusion broth, DD5025 grows noticeably slower in brain heart infusion broth containing 3.5% NaCl. One of the strategies used by bacteria to cope with environments with elevated osmolarity is to take up osmoprotective compounds, termed compatible solutes (60). gp59 is a member of the metabolite:H⁺ symporter family of transporters, and the metabolites transported by this family include compatible solutes (52). Thus, the biological function of gp59 may be to transport a compatible solute into the cell and allow *B. pseudomallei* 1026b to overcome environmental salt stress (60).

ϕ 1026b and ϕ E125 encode a RelE-like toxin that is flanked by a transcriptional regulator and a class I holin (Fig. 3). The presence of both a class I holin (gp82 in ϕ 1026b, gp70 in ϕ E125) and a class II holin (gp23 in ϕ 1026b, gp24 in ϕ E125) in these bacteriophages is unusual, and it is not known if one or both of these holins are required for the programmed release of lysozyme from the cytoplasm prior to the bacteriophage burst (65). RelE toxin and RelB antitoxin are members

of an *E. coli* toxin-antitoxin protein system that reversibly inhibits protein synthesis in response to nutrient limitation (33). The genes encoding toxin-antitoxin systems are widespread in bacteria and are typically adjacent to one another on plasmids or chromosomes (8). The antitoxin binds the toxin and prevents it from killing the bacterial host by binding to essential enzymes or disrupting important cellular functions. The antitoxin component is typically less stable than the toxin component, and decreased transcription or translation of the antitoxin results in death of the bacterial host. Toxin-antitoxin systems were first identified on plasmids, where they play an important role in plasmid stabilization (8). The presence of toxin-antitoxin genes in bacteriophage genomes is uncommon and may be a mechanism by which prophages maintain their genomes in their bacterial hosts. However, ϕ 1026b and ϕ E125 do not harbor an obvious antitoxin gene, and future experiments should explore if there is a novel antitoxin gene and what, if any, function the putative toxin-antitoxin system plays in these bacteriophages.

In conclusion, bacteriophages are significant contributors to the genomic diversity of *B. pseudomallei* isolates. The bacteriophage described in this study was specific for *B. mallei*, and it exhibited a mosaic genetic relationship with bacteriophage ϕ E125, another *B. mallei*-specific bacteriophage produced by *B. thailandensis* (70). Thus, it appears that *B. mallei* may be an ideal host for the study of additional bacteriophages produced by *B. pseudomallei* and *B. thailandensis*. Direct comparisons of the bacteriophages produced by these species may reveal virulence genes that are present in *B. pseudomallei* bacteriophages but not in *B. thailandensis* bacteriophages.

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